



Systematic ambiguity in the well-established model system insect *Scathophaga stercoraria* (Diptera: Scathophagidae): sister species *S. soror* revealed by molecular evidence

MARCO VALERIO BERNASCONI¹*, DAVID BERGER¹ & WOLF U. BLANCKENHORN¹

¹Zoological Museum, Institute of Evolutionary Biology and Environmental Studies, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

E-mails: marco.bernasconi@access.uzh.ch; david.berger@access.uzh.ch; wolf.blanckenhorn@ieu.uzh.ch

*corresponding author

Abstract

Even for well-established insect model systems, such as the yellow dung fly *Scathophaga stercoraria* (Linnaeus) (Diptera: Scathophagidae), there may be hidden systematic ambiguities that require clarification. Dung flies from the Afrotropical Region have been considered (i) as con-specific and not different from all the other Holarctic *Scathophaga stercoraria*; (ii) as a local and peculiar African subspecies of *S. stercoraria* (*Scathophaga stercoraria soror* Wiedemann), or (iii) as a separate valid species (*Scathophaga soror* Wiedemann). Our study represents an attempt, based on mitochondrial (COI, 12S, and 16S), nuclear (ITS2) as well as microsatellite markers, to clarify this problem. Results strongly suggest that *S. soror* is a separate taxon from *S. stercoraria*. Due to the importance of *S. stercoraria* as a model system for studies in ecology, behaviour and evolution, the systematic position of *S. soror* (relative to *S. stercoraria*) is not solely of interest for systematists, but for evolutionary ecologists as well.

Key words: Systematics, Phylogeny, Speciation, Cytochrome oxidase I, COI, 12S, 16S, ITS2, internal transcribed spacer 2, microsatellites

Introduction

Scathophagid flies, with about 400 species described, are mainly confined to the Holarctic Region and are more northern in overall distribution than any other family of Diptera (e.g., Sack 1937; Vockeroth 1987; but see also Šifner 2003, 2008, 2009). Only a few species occur in South Africa and at high altitudes in East Africa, the Andes, and the Oriental Region (see references cited in Bernasconi *et al.* 2000a). Many adult scathophagids are predators (e.g., on Simuliidae; Werner *et al.* 2006), and several Arctic species are regularly observed on carrion and mammalian dung. Some species breed in rotting seaweed. The eggs are attached to the leaf surface or inserted into plant tissues, dung, or other substrates. Most of the larvae are phytophagous, while others are carnivorous in dung or coprophagous (Gorodkov 1986; Vockeroth 1987; Šifner 2008). Individuals of most species thus perform the ecologically important function of resource recycling.

Within this family, the species *Scathophaga stercoraria* (Linnaeus) has served since the early 1960s in numerous ecological, behavioural and evolutionary investigations, particularly as model system for studies of sperm competition, cryptic female choice, and life history evolution (summarised in Ward 2007). The species is included in the first tier of the Flytree (Assembling the Diptera Tree of Life) project for this reason (<http://www.inhs.illinois.edu/research/FLYTREE/>).

From a morphological point of view there is no unanimity within the entomological community about the taxonomic status of the South African dung flies. They have been considered (i) as con-specific and not different from all the other *S. stercoraria* worldwide (e.g., Šifner 2008), (ii) as a peculiar subspecies (*Scathophaga stercoraria soror* Wiedemann, e.g., Vockeroth 1958), or even (iii) as a separate species (*Scathophaga soror* Wiedemann, e.g., Werner *et al.* 2006).

The relationships in this family are now generally well understood (Bernasconi *et al.* 2000a, 2000b, 2001; Kutty *et al.* 2007, 2008), but the systematic position (and identity) of *S. (stercoraria) soror* remains enigmatic, as this taxon has never been included in any molecular systematic or phylogenetic analysis of the family. Our study represents therefore an attempt, based on mitochondrial (COI, 12S, and 16S), nuclear (ITS2) as well as microsatellite markers, to clarify this problem.

Material and methods

Samples

A total of 50 specimens representing 21 species of Scathophagidae were included in the present study (Table 1). Whenever possible, various specimens belonging to the same species, but from different geographic origin, were included to gain information about the intra-specific genetic diversity of the species examined. In particular, six individuals of *S. (stercoraria) soror* from three different South African localities, as well as 14 specimens of *S. stercoraria* from Europe, Asia, and North America were included. Four *Gimnomera* species were used for outgroup comparison (see Bernasconi *et al.* 2000a; Kutty *et al.* 2007).

TABLE 1. Overview of samples and species of Scathophagidae used in this study.

Specimen voucher (sample id in Fig. 1 & Table 7)	Species	Origin	GenBank accession number			
			COI	12S	16S	ITS2
Bernasconi 0286 (P) (286)	<i>Ceratinostoma ostiorum</i> (Curtis, 1823)	United Kingdom	AF180792	DQ656914	DQ648668	EU884226
Bernasconi 0308 (P) (308)	<i>Gimnomera cerea</i> Coquillett, 1908	Old Chelsea, Quebec, Canada	AF181009	DQ656917	DQ648671	NA
Bernasconi 0227 (P) (227)	<i>Gimnomera cuneiventris</i> (Zetterstedt, 1846)	Abisko, Sweden	AF181011	DQ656918	DQ648672	NA
Bernasconi 0222 (P) (222)	<i>Gimnomera dorsata</i> (Zetterstedt, 1838)	Abisko, Sweden	AF181008	DQ656919	DQ648673	EU884220
Bernasconi 0291 (P) (291)	<i>Gimnomera tarsea</i> (Fallén, 1819)	Whitlaw Moss, United Kingdom	AF181010	DQ656920	DQ648674	EU884229
Bernasconi 0250 (P) (250)	<i>Scathophaga analis</i> (Meigen, 1826)	Prague, Czech Republic	AF180783	DQ656942	DQ648696	NA
Bernasconi 0287 (P) (287)	<i>Scathophaga calida</i> Curtis, 1832	United Kingdom	AF180787	DQ656943	DQ648697	EU884227
Bernasconi 0019 (P) (19)	<i>Scathophaga cineraria</i> (Meigen, 1826)	Switzerland	AF180784	DQ656944	DQ648698	EU884209
Bernasconi 0005 (P) (5)	<i>Scathophaga furcata</i> (Say, 1823)	Zurich, Switzerland	AF180775	NA	NA	EU884204
Bernasconi 0013 (P) (13)	<i>Scathophaga furcata</i>	Zurich, Switzerland	AF180776	NA	NA	EU884205
Bernasconi 0042 (P) (42)	<i>Scathophaga furcata</i>	Zurich, Switzerland	AF180777	DQ656945	DQ648699	EU884212
Bernasconi 0267 (P) (267)	<i>Scathophaga incola</i> (Becker, 1900)	Gronubakken, Norway	AF180786	DQ656946	DQ648700	EU884224
Bernasconi 0268 (P) (268)	<i>Scathophaga inquinata</i> (Meigen, 1826)	Hordaland, Norway	AF180781	DQ656947	DQ648701	EU884225

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TABLE 1 (continued)

Specimen voucher (sample id in Fig. 1 & Table 7)	Species	Origin	GenBank accession number			
			COI	12S	16S	ITS2
Bernasconi 0294 (P) (294)	<i>Scathophaga inquinata</i>	Finland	AF180782	NA	NA	EU884231
Bernasconi 0218 (P) (218)	<i>Scathophaga litorea</i> (Fallén, 1819)	Leuchtturm Kjolnes, Norway	AF180789	DQ656948	DQ648702	EU884219
Bernasconi 0038 (P) (38)	<i>Scathophaga lutaria</i> (Fabricius, 1794)	Zurich, Switzerland	AF180778	NA	NA	EU884210
Bernasconi 0046 (P) (46)	<i>Scathophaga lutaria</i>	Altbüron LU, Switzerland	AF180779	DQ656949	DQ648703	EU884214
Bernasconi 0108 (P) (108)	<i>Scathophaga lutaria</i>	Har Meron, Israel	AF180780	NA	NA	EU884215
Bernasconi 0293 (P) (293)	<i>Scathophaga obscura</i> (Fallén, 1819)	United Kingdom	AF180790	DQ656950	DQ648704	EU884230
Bernasconi 0266 (P) (266)	<i>Scathophaga pictipennis</i> (Oldenberg, 1923)	Kvam, Norway	AF180785	DQ656951	DQ648705	EU884223
Bernasconi 00S1 (P) (S1)	<i>Scathophaga soror</i> Wiedemann, 1818	Kwa Zulu-Natal, South Africa	EU884256	EU884234	EU884244	EU884192
Bernasconi 00S2 (P) (S2)	<i>Scathophaga soror</i>	Kwa Zulu-Natal, South Africa	EU884257	EU884235	EU884245	EU884193
Bernasconi 00S3 (P) (S3)	<i>Scathophaga soror</i>	Cape Province, South Africa	EU884258	EU884236	EU884246	NA
Bernasconi 00S4 (P) (S4)	<i>Scathophaga soror</i>	Cape Province, South Africa	EU884259	EU884237	EU884247	EU884194
Bernasconi 00S7 (P) (S7)	<i>Scathophaga soror</i>	Western Cape, South Africa	EU884262	EU884240	EU884250	EU884197
Bernasconi 00S8 (P) (S8)	<i>Scathophaga soror</i>	Western Cape, South Africa	EU884263	EU884241	EU884251	EU884198
Bernasconi 00S5 (P) (S5)	<i>Scathophaga stercoraria</i> (Linnaeus, 1758)	Hokkaido, Japan	EU884260	EU884238	EU884248	EU884195
Bernasconi 00S6 (P) (S6)	<i>Scathophaga stercoraria</i>	Hokkaido, Japan	EU884261	EU884239	EU884249	EU884196
Bernasconi 00S9 (P) (S9)	<i>Scathophaga stercoraria</i>	Lethbridge, Alberta, Canada	EU884264	NA	EU884252	NA
Bernasconi 0S10 (P) (S10)	<i>Scathophaga stercoraria</i>	Lethbridge, Alberta, Canada	EU884265	EU884242	EU884253	EU884199
Bernasconi 0S11 (P) (S11)	<i>Scathophaga stercoraria</i>	Lethbridge, Alberta, Canada	EU884266	EU884243	EU884254	EU884200
Bernasconi 0S12 (P) (S12)	<i>Scathophaga stercoraria</i>	Lethbridge, Alberta, Canada	EU884267	NA	EU884255	EU884201
Bernasconi 000C (P) (C)	<i>Scathophaga stercoraria</i>	Coldrerio TI, Switzerland	AF180759	NA	NA	EU884202
Bernasconi 000E (P) (E)	<i>Scathophaga stercoraria</i>	Aristau AG, Switzerland	AF180760	NA	NA	EU884203

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TABLE 1 (continued)

Specimen voucher (sample id in Fig. 1 & Table 7)	Species	Origin	GenBank accession number			
			COI	12S	16S	ITS2
Bernasconi 0011 (P) (11)	<i>Scathophaga stercoraria</i>	Zurich, Switzerland	AF180761	NA	NA	NA
Bernasconi 0039 (P) (39)	<i>Scathophaga stercoraria</i>	Zurich, Switzerland	AF180762	NA	NA	EU884211
Bernasconi 0044 (P) (44)	<i>Scathophaga stercoraria</i>	Altbüren LU, Switzerland	AF180763	DQ656952	DQ648706	EU884213
Bernasconi 0095 (P) (95)	<i>Scathophaga stercoraria</i>	Piora TI, Switzerland	AF180764	NA	NA	NA
Bernasconi 0295 (P) (295)	<i>Scathophaga stercoraria</i>	Wales (United Kingdom)	AF180765	NA	NA	EU884232
Bernasconi 0298 (P) (298)	<i>Scathophaga stercoraria</i>	Wales (United Kingdom)	AF180766	NA	NA	EU884233
Bernasconi 0015 (P) (15)	<i>Scathophaga suilla</i> (Fabricius, 1794)	Switzerland	AF180770	NA	NA	EU884206
Bernasconi 0016 (P) (16)	<i>Scathophaga suilla</i>	Switzerland	AF180771	NA	NA	EU884207
Bernasconi 0017 (P) (17)	<i>Scathophaga suilla</i>	Switzerland	AF180772	NA	NA	EU884208
Bernasconi 0207 (P) (207)	<i>Scathophaga suilla</i>	Origlio TI, Switzerland	AF180773	DQ656953	DQ648707	EU884218
Bernasconi 0249 (P) (249)	<i>Scathophaga suilla</i>	Zavistivý, Czech Republic	AF180774	NA	NA	NA
Bernasconi 0128 (P) (128)	<i>Scathophaga taeniopa</i> Rondani, 1866	Switzerland	AF180767	NA	NA	EU884216
Bernasconi 0179 (P) (179)	<i>Scathophaga taeniopa</i>	Val Bedretto TI, Switzerland	AF180768	DQ656954	DQ648708	EU884217
Bernasconi 0238 (P) (238)	<i>Scathophaga taeniopa</i>	Piora TI, Switzerland	AF180769	NA	NA	EU884221
Bernasconi 0263 (P) (263)	<i>Scathophaga tinctinervis</i> (Becker, 1894)	Whitlaw Moss, United Kingdom	AF180791	DQ656955	DQ648709	EU884222
Bernasconi 0289 (P) (289)	<i>Scathophaga tropicalis</i> Malloch, 1931	Bolivia	AF180788	DQ656956	DQ648710	EU884228

NA= Not Available.

DNA extraction, amplification, sequencing and microsatellites

DNA was extracted from fly specimens using a Dneasy Tissue kit (Qiagen AG, Hombrechtikon, Switzerland) carefully following the manufacturer's instructions. Entire specimens were first mechanically triturated in a microtube using a "TissueLyser" (Mixer Mill MM 300, Qiagen AG, Hombrechtikon, Switzerland). After digestion with Proteinase K (20µg/ml), samples were applied to the columns for absorption and to wash DNA. Finally, the DNA was eluted in 200µl of the buffer from the kit and stored at 4°C (Bernasconi *et al.* 2007a, 2007b). All the extracted specimens are deposited at the Zoological Museum, University of Zurich. Standard PCR reactions were performed with 2µl of the extracted DNA as template, 0,5µM of each primer, 1 Unit Taq Polymerase (HotStarTaq Master Mix Kit, Qiagen AG, Hombrechtikon,

Switzerland) in a total volume of 50µl (manufacturer's buffer). For all the gene fragments (COI, 12S, 16S, and ITS2), the reaction mixtures were subjected to 15 min DNA denaturation at 94°C, 35 cycles of denaturation at 94°C for 1 min, annealing at 48–54°C for 1 min, and elongation at 72°C for 2min. The elongation was completed by a further 7 min step at 72°C. The PCR reactions were performed in a DNA Thermal Cycler (Perkin-Elmer Applied Biosystems, Rotkreuz, Switzerland). The amplification and sequencing primers (Microsynth GmbH, Balgach, Switzerland) are listed in Table 2 (Bernasconi *et al.* 2000a, 2000b, 2001, Germann *et al.* 2009). Templates for direct sequencing were prepared by a simple purification step of PCR products using the QIAquick PCR Purification Kit (Qiagen AG, Hombrechtikon, Switzerland), or the NucleoSpin Extract II Kit (Macherey-Nagel AG, Oensingen, Switzerland), following in both cases the manufacturer's instructions. Alternatively, the purification of the PCR products was performed by adding to each PCR product 2ml (1U/ml) Shrimp Alkaline Phosphatase (Promega AG, Wallisellen, Switzerland) and 1ml (20U/ml) Exonuclease I (New England Biolabs (Bioconcept), Allschwil, Switzerland). The ExoSAP protocol consisted of 45 min incubation at 37°C and 15 min deactivation at 80°C. Cycle sequencing reactions were performed in total volumes of 15ml using an ABI Prism Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Rotkreuz, Switzerland), purified by using DyeEx 2.0 Spin Kit (Qiagen AG, Hombrechtikon, Switzerland) or NucleoSEQ Kit (Macherey-Nagel AG, Oensingen, Switzerland), on an ABI Prism 3100 Avant Genetic Analyser (Perkin-Elmer Applied Biosystems) or on an ABI 3730 DNA Analyser (Perkin-Elmer Applied Biosystems), again following the manufacturer's instructions.

TABLE 2. Amplification and sequencing primers used.

Target region	Primer	Strand	Size (nt)	Sequence 5'-3'
COI gene	C1-J-2183TT	Major	23	CAACATTTATTTTGATTTTTTGG
	TL2-N-3014	Minor	25	TCCATTGCACTAATCTGCCATATTA
12S gene	SR-J-14233	Major	20	AAGAGCGACGGGCGATGTGT
	SR-N-14588	Minor	25	AAACTAGGATTAGATACCCTATTAT
16S gene	LR-J-12887	Major	22	CCGGTTTGAAGTCAGATCATGT
	LR-N-13398	Minor	20	CGCCTGTTTAACAAAAACAT
ITS2 region	ITS-02	Forward	21	TGGGTCGATGAAGAACGCAGC
	ITS-04	Reverse	22	TCCTTGTTAGTTTCTTTTCCTC

The 12 microsatellite primers (Microsynth GmbH, Balgach, Switzerland) and the related protocols used here are reported in detail in Garner *et al.* (2000), Watts *et al.* (2005), Demont *et al.* (2008) and Bussière *et al.* (2010) and are listed in Table 3. In summary, the Qiagen Multiplex PCR kit (Qiagen AG, Hombrechtikon, Switzerland) was used for the amplification process. Total PCR reaction volume was 6µl: 1µl DNA template, 3µl Qiagen Multiplex PCR Master Mix, 1.4µl distilled water and 0.6µl microsatellite primer mix (100µM). For the dinucleotides microsatellite primers, the reaction mixtures were subjected to 15 min DNA denaturation at 94°C, 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 3 min, and elongation at 72°C for 45 sec. The elongation was completed by a further 30 min step at 60°C. For the trinucleotides microsatellite primers, the reaction mixtures were subjected to 15 min DNA denaturation at 94°C, 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 3 min, and elongation at 72°C for 45 sec. The elongation was completed by a further 30 min step at 60°C. PCR products were separated on a capillary sequencer (Perkin-Elmer Applied Biosystems ABI 3730 DNA Analyser), and the output was analysed using Applied Biosystems GeneMapper software (Perkin-Elmer Applied Biosystems, Rotkreuz, Switzerland).

TABLE 3. Primer sequences for the *Scathophaga stercoraria* microsatellite loci used in this study.

Primer Name	Size (nt)	Primer Sequence 5'-3'
Dinucleotides		
SsCa1F	20	TCC TGG GCC ATA ATC ACA AC
SsCa1R	20	TGG TCC TTT GGA CTT GTG TG
SsCa3F	19	CCT CAA CCC CCT CAC TCA C
SsCa3R	27	CAT CAT CAT TTA AGT CAA CAT TAG AAA
SsCa16F	20	TTG GCG TCA CCA TAC TCA AC
SsCa16R	21	GAC TTT GGT CCG TTG TAG TCC
SsCa21F	20	TGG CTG GGA ATC TCA TGT CT
SsCa21R	21	TGT GGC ATC AAA AAT CAA CAA
SsCA24F	20	CAC ACA CTC GCA GCT ACA CC
SsCA24R	24	AAA CTT TAA CTT CGA TTT TTG CTG
SsCA26F	18	CAG CAA AAA CCG GCA AAC
SsCA26R	19	TGC CAC TTT TGG TGC TTT C
SsCA30F	22	TGC GAA AAA GTC TCA TAA CTC G
SsCA30R	27	GAT GAA ATA TGT ATG CAT GTG TTA GTT
Trinucleotides		
SsTri-04F	23	CAG CAC ATT CAA CAA CTG CAA CA
SsTri-04R	20	CCG CCA CCA CTG TCA TCA AC
SsTri-11F	18	GCT CAA CCG CAC AAT CAG
SsTri-11R	20	AAT GAA GCC GAT GCT CTG TT
SsTri-12F	20	GTC GTC GTG CAA TTA CTC TT
SsTri-12R	20	CTA CAG TCG TGC ATT ACG TT
SsTri-18F	20	AAT AAT GTG CTG ATG GCT GC
SsTri-18R	20	CTA ATA CAA CAA CCA CCA CC
SsTri-20F	20	ACT AAT GGT GGT GGT GTA GG
SsTri-20R	20	AGG GCT GAT GAT GAT GCT GC

DNA sequence analyses

The genetic sequences (COI, 12S, 16S, and ITS2) were handled and stored with the Lasergene program Editseq (DNASTar Inc., Madison, WI USA). Alignment of all gene sequences was performed using Megalign (DNASTar Inc.) with default multiple alignment parameters (“gap penalty=15”; “gap length penalty=6.66”; “delay divergent sqs(%)=30”; “DNA transition weight=0.50”). The COI alignment was gap-free. The alignment of the 16S, 12S, and ITS2 fragments was usually satisfactory enough with the default parameters and did not require particular manual interventions. However, when necessary, gaps were manually included to allow a better and correct alignment of the homologous corresponding regions. ForCon (Raes & Van de Peer 1999), a software tool for the format conversion of sequence alignments, was further applied. The partition-homogeneity test (ILD test, Farris *et al.* 1994) implemented in PAUP*4.0b10 (Swofford 2002) was used to test whether datasets could be combined.

Phylogenetic reconstruction was carried out using Bayesian analysis, performed with MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). Modeltest 3.5 (Posada & Crandall 1998) was used to identify the

evolutionary model(s) appropriate for the Bayesian analyses. Thus, the data were partitioned by gene (COI, 12S, 16S, and ITS2), and the COI gene was further partitioned by codon (first-, second-, and third-codon position). Bayesian analyses (finally based on a data set comprising only one specimen for each species; see Results) were allowed to use a mixed model (i.e., a model in which all genes have their unique GTR+I+G model), and the Markov chain Monte Carlo search was run with 4 chains (one cold and three heated) for 1,000,000–1,500,000 generations, with trees being sampled every 100 generations. The heating of the chains was adjusted to get the acceptance rates for the swaps between chains to 10–70% (the “temp” parameter varied therefore from 0.05 to 0.3). Various independent trials were performed on two different computers. To determine the “burn-in”, log-likelihood plots were examined for stationarity (where plotted values reach an asymptote). In all analyses, stationarity was clearly reached after less than 100,000 generations (=1000 trees), but we discarded the first 2000–3000 trees to ensure that it was completely reached. Higher “burn-in” did not alter the topology of the final 50% majority rule consensus tree(s). Bayesian posterior probabilities were therefore given by the percentage of runs that produced each branch and were calculated from the remaining trees generated from the two parallel runs. In all analyses, the two independent runs executed in parallel always converged, reaching average standard deviation values for the split frequencies of less than 0.05. Preliminary analyses (involving the single genes as well as the combined dataset) using the Maximum Parsimony and the Neighbour Joining method were performed with MEGA (Molecular Evolutionary Genetics Analysis version 4; Tamura *et al.* 2007) and PAUP*4.0b10. The sequences of the three mitochondrial gene fragments and the ITS2 region for the 50 Scathophagidae specimens analysed here have been deposited in GenBank (Table 1).

Results

The partition homogeneity test indicated that the four gene partitions were not significantly mutually incongruent ($p=0.06$ for all gene partitions; $p=0.28$ for mtDNA *vs* ITS2), which justified the combination of the four data sets. Even if $p=0.06$ is close to significant heterogeneity, a significance threshold of 0.05 may be too conservative for the ILD test according to Sullivan (1996). Moreover, as stated by Cunningham (1997), whenever the ILD test finds a p -value greater than 0.01, combining the data improve or at least do not reduce phylogenetic accuracy. All reported results therefore are based on the total molecular evidence resulting from the concatenation of the four partitions. The full data set comprises, including indels, 2531 characters (COI: 810; 12S: 650; 16S: 512; ITS2: 559) with 441 variable sites (COI: 217; 12S: 65; 16S: 33; ITS2: 126).

All the specimens formally belonging to the same (recognised) species clustered together. The taxa therefore proved to be monophyletic in all the preliminary analyses performed involving all the specimens available belonging to the same (recognised) species. Consequently, only one specimen from each species was included in the final phylogenetic analyses. Phylogenetic relationships derived from 18002 Bayesian trees (9001 trees for each of the two parallel runs) based on combined COI, 12S, 16S, and ITS2 sequences as established for 21 Scathophagidae species are illustrated in Figure 1. Tables 4 and 5 summarise, respectively, the intra- and the inter-specific genetic distances (uncorrected p -distance) recorded for the four gene fragments sequenced here (intra-specific genetic distance only for the species represented by more than one specimen). The intra-specific uniformity of the *S. soror* and *S. stercoraria* samples contrasts with the inter-specific genetic distance observed between the two taxa. Using the genetic distance between *S. soror* and *S. stercoraria* (0.018) as a yardstick, this value is often higher than those between well-established and universally recognised species (all genetic distances <0.018 are evidenced in bold in Table 5). Table 6 illustrates the characteristic point mutations or indels (“species diagnostic substitutions”) in the COI, 12S, 16S, and ITS2 sequences distinguishing between *S. soror* and *S. stercoraria* specimens. Based on our molecular sequence data, *S. soror* is clearly a separate taxon and the sister species of *S. stercoraria* (Fig. 1). This result is also corroborated by the microsatellite data (Table 7). Not all the primers for the microsatellite loci developed for *S. stercoraria* amplify in the *S. soror* samples (or the other scathophagid species examined here). In particular, only the “Trinucleotide microsatellite locus 12” (SsTri-12) is specific for *S. stercoraria*.

and does not cross-amplify in *S. soror*. Thus the microsatellite data also suggest that the fly specimens from South Africa are a separate taxon from all the *S. stercoraria* populations from around the world (including Europe, Canada, and Japan).

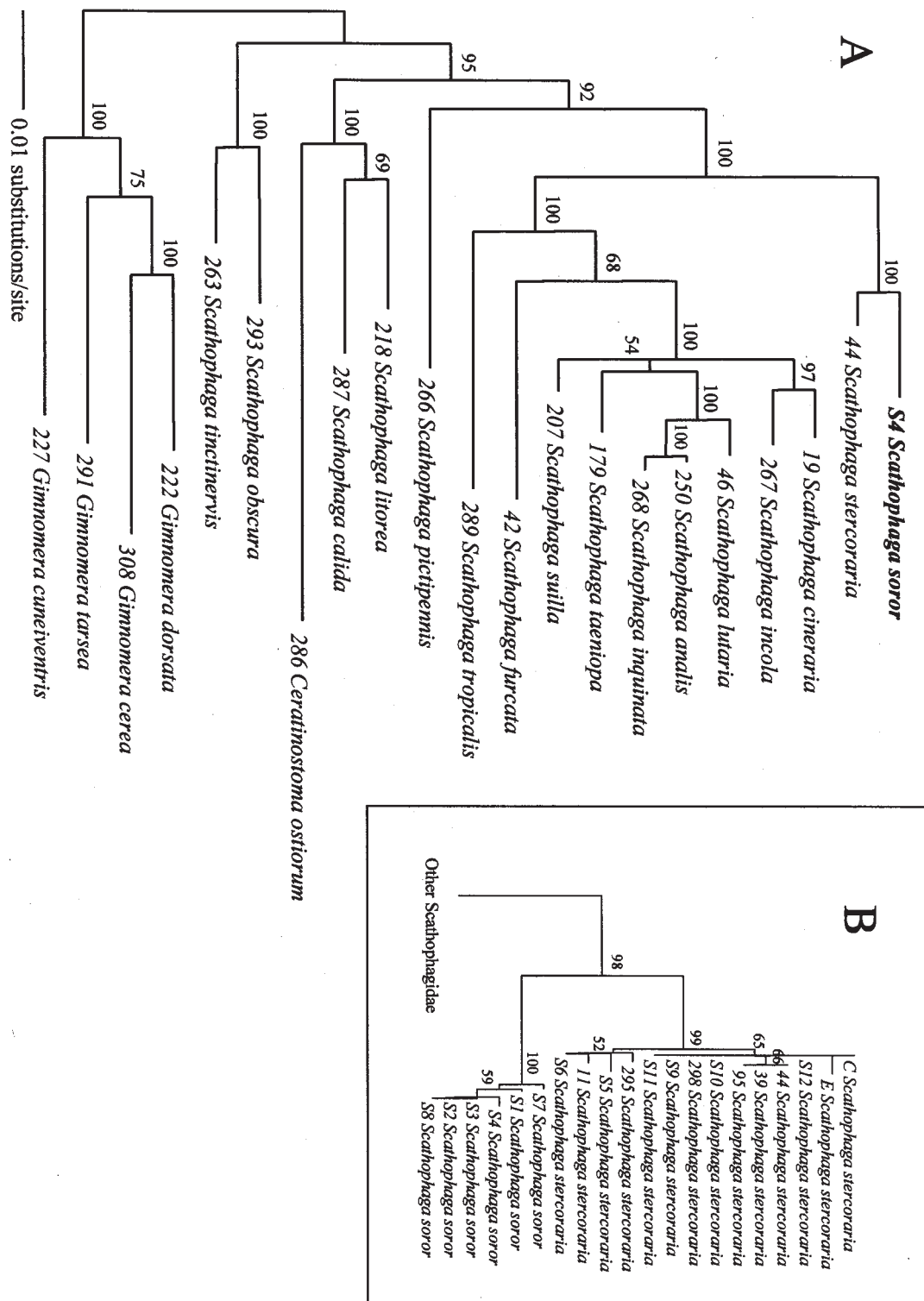


FIGURE 1. A. Phylogenetic relationships derived from 18'002 Bayesian trees based on combined COI, 12S rDNA, 16S rDNA, and ITS2 sequences as established between 21 Scathophagidae species. The tree is a 50% majority rule consensus tree; values of posterior probabilities over 50% are indicated above branches (branches with probabilities less than 50% are collapsed). *Scathophaga soror* is evidenced in bold. **B.** Excerpt from a gene tree (Neighbour Joining, Kimura 2 parameters, COI gene) illustrating the sister group relationship between the monophyletic *S. stercoraria* and *S. soror* clades. Bootstrap values (for 1000 pseudo-replicates) are indicated above branches.

TABLE 4. Maximal intra-specific genetic distances (uncorrected p-distance) recorded for the four genes sequenced and for the species represented by more than one specimen.

	COI	12S	16S	ITS2
<i>S. soror</i>	0.003	0	0	0
<i>S. stercoraria</i>	0.007	0	0	0
<i>S. furcata</i>	0.004	NA	NA	0.002
<i>S. inquinata</i>	0.002	NA	NA	0.002
<i>S. lutaria</i>	0	NA	NA	0
<i>S. suilla</i>	0.004	NA	NA	0
<i>S. taeniopa</i>	0	NA	NA	0

NA= Not Available.

Discussion

Our results based on mitochondrial and nuclear DNA sequences as well as microsatellites especially designed to differentiate among *S. stercoraria* populations (Garner *et al.* 2000; Watts *et al.* 2005; Demont *et al.* 2008) support the hypothesis that dung fly specimens from South Africa are a separate taxon from all *S. stercoraria* populations from Europe, North America, and Asia analysed so far. But are *S. soror* and *S. stercoraria* two different species (or subspecies)? Being aware of the potential weaknesses of determining species solely on the basis of genetic distances (Bernasconi *et al.* 2007a; Germann *et al.* 2009), in this case several lines of evidence seem to support the idea that these taxa should be considered separate species (or at least subspecies). The uniformity in the genetic distances recorded within *S. stercoraria* populations from around the world, together with the similarly homogeneous genetic distances recorded among the *S. soror* samples from throughout South Africa, markedly contrasts with the genetic distance observed in the *S. soror*–*S. stercoraria* pair. The genetic distance recorded between *S. soror* and *S. stercoraria* is in many cases larger than that observed among well-defined and universally accepted species (Table 5). More importantly, there are a number of species diagnostic substitutions in both the mitochondrial and the nuclear ITS2 sequences that allow clear discrimination between the two taxa (Table 6). In addition, all the primers for the microsatellites designed for *S. stercoraria* function well (as expected) for all the populations of this species but not all primers amplify in the *S. soror* specimens (Table 7). Therefore, based on our genetic data, we strongly suggest the treatment of *S. soror* as a distinct species from *S. stercoraria*.

From a morphological point of view, the situation is rather unclear. On the one hand, Werner *et al.* (2006) state that “males of *S. stercoraria* are very variable in appearance, and may be large and covered with long dense bright yellow hairs, or smaller, duller, yellowish-grey to grey and with less dense hairs. Superficially, *soror* resembles a less robust and less hairy form of *stercoraria*, but there are differences in the male genitalia between European males (U. K.) and South African males (5 males dissected) which suggest that these two should be ranked as good species” (p. 147). Similarly, Vockeroth (1958) showed that *S. soror* differs from the typical *S. stercoraria* by subtle and not entirely consistent characters of colour and bristling and therefore considered *S. soror* as a subspecies of *S. stercoraria*. On the other hand, Šifner (2008) considered all characters of *S. soror* within the limits of variability of *S. stercoraria* and regarded *S. soror* as a synonym of *S. stercoraria* (Šifner 2008). A detailed morphological comparison of male terminalic structures (especially the pregonite; Šifner, pers. comm.) in both *S. soror* and *S. stercoraria* specimens should clarify the distinction on purely morphological grounds. However, it cannot be excluded that both dung flies (the African *S. soror* and the rather cosmopolitan *S. stercoraria*) co-exist in the Afrotropical Region. Examination of (older) museum specimens as well as newly sampled exemplars in South and East Africa would help much to clarify this question.

TABLE 5. Inter-specific genetic distances (uncorrected p-distance) recorded in the combined COI, 12S, 16S, and ITS2 data set as established between 21 Scathophagidae species

	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]
[1]																				
[2]	0.034																			
[3]	0.034	0.025																		
[4]	0.018	0.025	0.032																	
[5]	0.035	0.015	0.026	0.030																
[6]	0.036	0.020	0.029	0.029	0.020															
[7]	0.029	0.012	0.026	0.022	0.017	0.014														
[8]	0.037	0.035	0.037	0.032	0.038	0.038	0.030													
[9]	0.060	0.059	0.057	0.055	0.063	0.063	0.059	0.054												
[10]	0.057	0.056	0.063	0.054	0.059	0.059	0.053	0.059	0.055											
[11]	0.036	0.034	0.043	0.033	0.036	0.038	0.032	0.032	0.037	0.057										
[12]	0.034	0.015	0.026	0.029	0.010	0.019	0.016	0.038	0.059	0.054	0.034									
[13]	0.037	0.028	0.037	0.028	0.031	0.032	0.026	0.026	0.054	0.058	0.023	0.030								
[14]	0.033	0.034	0.039	0.032	0.039	0.042	0.032	0.036	0.059	0.058	0.037	0.035	0.034							
[15]	0.035	0.015	0.028	0.029	0.018	0.015	0.012	0.037	0.062	0.058	0.036	0.019	0.032	0.039						
[16]	0.034	0.015	0.026	0.029	0.010	0.019	0.016	0.038	0.059	0.054	0.033	0.001	0.030	0.035	0.019					
[17]	0.064	0.063	0.071	0.064	0.065	0.063	0.060	0.067	0.081	0.072	0.065	0.063	0.064	0.065	0.068	0.063				
[18]	0.041	0.038	0.045	0.037	0.044	0.043	0.037	0.039	0.060	0.059	0.040	0.041	0.040	0.046	0.041	0.041	0.064			
[19]	0.044	0.028	0.038	0.041	0.034	0.038	0.030	0.044	0.065	0.061	0.044	0.035	0.042	0.045	0.032	0.035	0.072	0.048		
[20]	0.063	0.059	0.064	0.059	0.063	0.067	0.063	0.055	0.049	0.057	0.061	0.061	0.057	0.061	0.064	0.061	0.084	0.068	0.063	
[21]	0.061	0.057	0.061	0.056	0.057	0.057	0.054	0.060	0.050	0.057	0.056	0.055	0.050	0.056	0.058	0.055	0.084	0.066	0.066	0.059

Numbers refer to the following species: [1] *Scathophaga soror*, [2] *Scathophaga cineraria*, [3] *Scathophaga fuscata*, [4] *Scathophaga stercoraria*, [5] *Scathophaga lutaria*, [6] *Scathophaga taeniopa*, [7] *Scathophaga suilla*, [8] *Scathophaga litorea*, [9] *Gimnomera dorsata*, [10] *Gimnomera cuneiventris*, [11] *Scathophaga obscura*, [12] *Scathophaga analis*, [13] *Scathophaga tinctinervis*, [14] *Scathophaga pictipennis*, [15] *Scathophaga incola*, [16] *Scathophaga inquinata*, [17] *Ceratinostoma ostiorum*, [18] *Scathophaga calida*, [19] *Scathophaga tropicalis*, [20] *Gimnomera tarsea*, [21] *Gimnomera cerea*.

The genetic distance between *S. soror* and *S. stercoraria* is evidenced in both **bold** and *italics*; genetic distances < 0.018 are evidenced in **bold** only.

TABLE 6. Variable sites between *S. stercoraria* and *S. soror* specimens recorded in COI, 12S, 16S, and ITS2 sequences. These characteristic point mutations or indels (“species diagnostic substitutions”) allow a clear distinction between the two species.

COI																					12S		16S				ITS2		
Position	0	1	1	2	3	3	3	3	4	4	4	4	5	5	5	5	6	6	6	7	3	5	0	2	2	2	3	4	4
	3	3	3	3	3	5	7	9	0	2	4	5	0	5	8	9	0	3	4	1	8	2	4	3	6	7	8	2	3
	3	5	8	5	6	7	8	3	6	6	7	6	7	5	5	4	9	6	5	7	3	1	2	6	6	4	0	2	0
<i>S. stercoraria</i>	T	T	C	T	T	T	C	T	T	C	A	C	T	C	T	T	A	A	T	T	A	C	A	A	G	T	C	T	C
<i>S. soror</i>	C	C	T	C	C	C	T	C	C	T	T	T	C	T	C	C	T	G	C	C	T	T	T	T	A	C	T	-	T

TABLE 7. Overview of the amplification success of the tri- and di-nucleotide microsatellite loci in the Scathophagid species used in this study.

sam- ple id	species	Origin	Trinucleotides microsatellite locus (SsTri-)					Dinucleotides microsatellite locus (SsCA)						
			4	11	12	18	20	1	3	16	21	24	26	30
286	<i>Ceratinostoma ostiorum</i>	United Kingdom	NA	NA	NA	NA	NA	0	0	1	0	1	1	0
308	<i>Gimnomera cerea</i>	Old Chelsea, Quebec, Canada	1	1	0	1	1	0	0	1	0	0	1	0
227	<i>Gimnomera cuneiventris</i>	Abisko, Sweden	1	1	0	1	0	0	0	1	0	0	1	0
222	<i>Gimnomera dorsata</i>	Abisko, Sweden	1	0	0	0	0	0	0	1	0	1	0	0
291	<i>Gimnomera tarsea</i>	Whitlaw Moss, UK	NA	NA	NA	NA	NA	0	0	1	0	0	1	0
250	<i>Scathophaga analis</i>	Prague, Czech Republic	1	1	0	0	1	0	0	1	0	1	1	0
287	<i>Scathophaga calida</i>	United Kingdom	1	1	0	0	1	0	0	1	0	0	1	0
19	<i>Scathophaga cineraria</i>	Switzerland	1	1	0	1	1	0	0	1	0	1	1	0
5	<i>Scathophaga furcata</i>	Zurich, Switzerland	NA	NA	NA	NA	NA	0	0	1	0	1	1	0
13	<i>Scathophaga furcata</i>	Zurich, Switzerland	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
42	<i>Scathophaga furcata</i>	Zurich, Switzerland	NA	NA	NA	NA	NA	0	0	1	0	1	1	0
267	<i>Scathophaga incola</i>	Gronubakken, Norway	1	1	0	0	1	0	0	1	0	1	1	0
268	<i>Scathophaga inquinata</i>	Hordaland, Norway	1	1	0	0	1	NA	NA	NA	NA	NA	NA	NA
294	<i>Scathophaga inquinata</i>	Finland	1	1	0	0	1	0	0	1	0	1	1	0
218	<i>Scathophaga litorea</i>	Leuchtturm Kjolnes, Norway	1	1	0	0	0	0	0	1	0	1	1	0
38	<i>Scathophaga lutaria</i>	Zurich, Switzerland	1	1	0	0	1	0	0	1	0	1	1	0
46	<i>Scathophaga lutaria</i>	Altbüron, Switzerland	NA	NA	NA	NA	NA	0	0	1	0	1	1	0
108	<i>Scathophaga lutaria</i>	Har Meron, Israel	1	1	0	0	1	0	0	1	0	1	1	0
293	<i>Scathophaga obscura</i>	United Kingdom	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
266	<i>Scathophaga pictipennis</i>	Kvam, Norway	1	1	0	0	1	0	0	1	0	0	1	0
S1	<i>Scathophaga soror</i>	Kwa Zulu-Natal, South Africa	1	1	0	1	1	1	1	1	1	1	1	1
S2	<i>Scathophaga soror</i>	Kwa Zulu-Natal, South Africa	1	1	0	1	1	1	1	1	1	1	1	1
S3	<i>Scathophaga soror</i>	Cape Province, South Africa	1	1	0	1	1	1	1	1	1	1	1	1
S4	<i>Scathophaga soror</i>	Cape Province, South Africa	1	1	0	1	1	1	1	1	1	1	1	1
S7	<i>Scathophaga soror</i>	Western Cape, South Africa	1	1	0	1	1	1	1	1	1	1	1	1
S8	<i>Scathophaga soror</i>	Western Cape, South Africa	1	1	0	1	1	1	1	1	1	1	1	1
11	<i>Scathophaga stercoraria</i>	Zurich, Switzerland	1	1	1	1	1	1	1	1	1	1	1	1
39	<i>Scathophaga stercoraria</i>	Zurich, Switzerland	1	1	1	1	1	1	1	1	1	1	1	1

.....continued on the next page

TABLE 7 (continued)

sam- ple id	species	Origin	Trinucleotides microsatellite locus (SsTri-)					Dinucleotides microsatellite locus (SsCA)						
			4	11	12	18	20	1	3	16	21	24	26	30
44	<i>Scathophaga stercoraria</i>	Altbüren, Switzerland	1	1	1	1	1	1	1	1	1	1	1	1
95	<i>Scathophaga stercoraria</i>	Piora, Switzerland	1	1	1	1	1	1	1	1	1	1	1	1
295	<i>Scathophaga stercoraria</i>	Wales (United Kingdom)	1	1	1	1	1	1	1	1	1	1	1	1
298	<i>Scathophaga stercoraria</i>	Wales (United Kingdom)	1	1	1	1	1	1	1	1	1	1	1	1
C	<i>Scathophaga stercoraria</i>	Coldrerio, Switzerland	1	1	1	1	1	1	1	1	1	1	1	1
E	<i>Scathophaga stercoraria</i>	Aristau, Switzerland	1	1	1	1	1	1	1	1	1	1	1	1
S10	<i>Scathophaga stercoraria</i>	Lethbridge, Alberta, Canada	1	1	1	1	1	1	1	1	1	1	1	1
S11	<i>Scathophaga stercoraria</i>	Lethbridge, Alberta, Canada	1	1	1	1	1	1	1	1	1	1	1	1
S12	<i>Scathophaga stercoraria</i>	Lethbridge, Alberta, Canada	1	1	1	1	1	1	1	1	1	1	1	1
S5	<i>Scathophaga stercoraria</i>	Hokkaido, Japan	1	1	1	1	1	1	1	1	1	1	1	1
S6	<i>Scathophaga stercoraria</i>	Hokkaido, Japan	1	1	1	1	1	1	1	1	1	1	1	1
S9	<i>Scathophaga stercoraria</i>	Lethbridge, Alberta, Canada	1	1	1	1	1	1	1	1	1	1	1	1
15	<i>Scathophaga suilla</i>	Switzerland	1	1	0	0	1	0	0	1	0	1	1	0
16	<i>Scathophaga suilla</i>	Switzerland	1	1	0	0	1	0	0	1	0	1	1	0
17	<i>Scathophaga suilla</i>	Switzerland	1	1	0	0	1	0	0	1	0	1	1	0
207	<i>Scathophaga suilla</i>	Origlio, Switzerland	NA	NA	NA	NA	NA	0	0	1	0	1	1	0
249	<i>Scathophaga suilla</i>	Zavistivý, Czech Republic	NA	NA	NA	NA	NA	0	0	1	0	1	1	0
128	<i>Scathophaga taeniopa</i>	Switzerland	1	1	0	0	1	0	0	1	0	1	1	0
179	<i>Scathophaga taeniopa</i>	Val Bedretto, Switzerland	1	1	0	0	1	NA	NA	NA	NA	NA	NA	NA
238	<i>Scathophaga taeniopa</i>	Piora, Switzerland	1	1	0	0	1	0	0	1	0	1	1	0
263	<i>Scathophaga tinctinervis</i>	Whitlaw Moss, UK	1	1	0	0	1	0	0	1	0	1	1	0
289	<i>Scathophaga tropicalis</i>	Bolivia	1	1	0	0	1	0	1	1	0	1	1	0
Sum of scores			41	40	14	23	38	20	21	45	20	40	44	20

=Not Available

In closing we would like to point out that the systematic position of *S. soror* (with respect to *S. stercoraria*) is not solely of interest for systematists. Since *S. stercoraria* is a widely used model organism in various areas of (evolutionary) biological research, the extension of the *S. stercoraria* model with *S. soror* may provide evolutionary biologists with a unique and powerful tool for studying speciation mechanisms in the context of sexual selection aspects such as sperm competition and cryptic female choice. Hybridisation experiments between the two taxa will therefore not only help understand this specific taxonomic issue, but will represent an opportunity for studying evolution in a model system involving sister taxa that have recently undergone or are undergoing speciation.

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